genes was reported in mouse myelomas (Honjo & Kataoka, 1978).

Since the γ-chain gene sequences did not show any homology with other class (α and μ) gene sequences, early divergence of classes of immunoglobulins must have occurred. This observation agrees with phylogenetic evidence that lower vertebrates such as sharks appear to have only IgM and amphibians have IgM and IgG, although all these classes in addition to IgA commonly exist in mammalians (Fudenberg et al., 1972). The emergence of subclasses is probably a result of recent evolution because the members of the γ-chain gene share considerable homology with each other. Pink et al. (1971) have concluded that subclasses in several species have evolved after differentiation of species. The amino acid sequence studies on γ1 chain (Milstein et al., 1974) and γ2a chain (Fougereau et al., 1976), which are the only two cases that the complete amino acid sequences of mouse immunoglobulin heavy chains are determined, showed that 62% of the C region sequences of two chains is homologous. Our estimation that nucleotide sequence homology between the γ1 and γ2a genes is about 62% (Table II) is in agreement with the amino acid sequence data.

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Cell-Free Translation of Mammalian Myosin Heavy-Chain Messenger Ribonucleic Acid from Growing and Fused-L_6E_9 Myoblasts†

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ABSTRACT: An mRNA-dependent reticulocyte cell-free protein synthesizing system very efficient in the translation of myosin heavy-chain mRNA from a rat myogenic cell line is described. This system exhibits a high degree of fidelity with regard to the spectrum and relative proportion of the different proteins synthesized from a sample of cytoplasmic RNA as compared to the proteins synthesized in vivo by the cells from which the RNA is prepared. The main feature of this system is the use of a K^+ and Cl^- concentration similar to those of the reticulocyte cytoplasm. Using this system, myosin heavy chain, identified by low-salt precipitation, electrophoretic mobility, and partial peptide analysis, represents 17% of the total protein synthesis when cytoplasmic RNA from well-fused L_6E_9 cells is used. Furthermore, when RNA preparations from growing myoblasts, that when analyzed in other cell-free translational systems seem not to contain any myosin heavy-chain mRNA, are tested in the system reported here, they are proven to contain high amounts of translatable myosin heavy-chain mRNA.

One of the most efficient eukaryotic systems for studying protein synthesis in vitro is the exogeneous mRNA-dependent unfractionated reticulocyte lysate (Hunt & Jackson, 1974; Pelham & Jackson, 1976). In vitro protein synthesizing systems provide a biological assay for the presence of functional mRNAs for specific proteins. By employing identical RNA concentrations from cultures at different stages of differentiation, it is possible to obtain an estimate of the amount of translatable mRNA for a specific protein present at one stage of differentiation relative to another. Such experiments can be coupled with in vivo studies on the accumulation of specific proteins during the course of differentiation to determine whether the controls on the synthesis of these proteins occur at a translational or a pretranslational level.

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We were interested in determining whether changes in myosin heavy-chain (MHC) accumulation during differentiation of myogenic cell lines in vitro were correlated with parallel changes in MHC mRNA accumulation. MHC is a 200000-dalton protein that is relatively easy to identify by its mobility on polyacrylamide gels and by its physical properties. The mRNA coding for chicken MHC has been assayed in reticulocyte lysate cell-free systems during the course of myogenic differentiation (Strohman et al., 1977). However, animal mRNAs specifying proteins of molecular weight greater than 100000, such as MHC, are translated very poorly. Under conditions in which large mRNAs are more effectively translated, total protein synthesis is drastically reduced or altered (Alton & Lodish, 1977; Pelham & Jackson, 1976). Furthermore, even under conditions optimal for the translation of mRNAs specifying proteins of molecular weight greater than 100000, the amount of MHC synthesized was not always directly proportional to the amount of RNA added to the translation reaction (Strohman et al., 1977). Under such conditions, it would be impossible to compare the relative amounts of MHC mRNA present at one time during differentiation relative to another. We report here on the development of a highly efficient mRNA-dependent reticulocyte lysate in vitro system that authentically translates the mRNAs for high molecular weight proteins while at the same time keeping total protein synthesis at a maximum. This system exhibits a high degree of fidelity with regard to the spectrum and the relative proportions of the different proteins synthesized from a sample of total cytoplasmic RNA as compared to the proteins synthesized in vivo by the cells from which the RNA was prepared. Furthermore, when RNA preparations from growing myoblasts, that when analyzed in other in vitro translational systems seem not to contain any MHC mRNA, are tested in the system reported here they are proven to contain high amounts of translatable MHC mRNA.

Materials and Methods

Cell Lines, Cell Culture, and Differentiation Conditions. L_6E_9 is a subclone of the L_6 rat myogenic cell line (Yaffe, 1968) isolated in our laboratory. Myoblast cell cultures were maintained in DME medium (Gibco) supplemented with 20% fetal calf serum (Gibco). For induction of myogenic differentiation, exponentially growing cultures were transferred to DME supplemented with 10% horse serum (Gibco). All cultures were incubated at 37 °C in a humidified 10% CO_2 atmosphere in air.

Isolation of RNA. Total cytoplasmic RNA was isolated using guanidine hydrochloride following the procedure of Cox (1968) with the following modification: cells were lysed with 0.5% NP-40 in a high-salt buffer (250 mM NaCl, 5 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.4) to prevent the precipitation of polysomal MHC mRNA. Purified RNA was stored as an ethanol precipitate at −60 °C until used.

Oligo(dT)-cellulose (Collaborative Research) chromatography was performed according to the method of Aviv & Leder (1972). RNA which bound to the column [poly(A)$^+$ RNA] as well as RNA which did not bind to the column was stored at −60 °C as ethanol precipitates.

Total cell polysomes were prepared from the postmitochondrial supernatant (PMS) of cells lysed in high-salt buffer. The PMS was layered over a 5-mL pad of 1 M sucrose in 100 mM NaCl, 5 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.4, and centrifuged at 40000 rpm for 150 min in a Beckman SW 41 rotor. The polysomal pellet was rinsed once with 100 mM NaCl, 5 mM $MgCl_2$, 10 mM Tris, pH 7.4, to remove the sucrose and stored in liquid nitrogen vapor until used.

Preparation of Reticulocyte Lysate. Rabbit reticulocyte lysates were prepared according to the procedure of Schimke et al. (1974) except that blood was collected by cardiac puncture into 50-mL centrifuge tubes containing 1500 units of sodium heparin. Lysates were stored in 400–450-μL aliquots in liquid nitrogen.

Cell-Free Protein Synthesis. Ethanol precipitates of RNA were collected by centrifugation at 16300*g* for 20 min. The pelleted RNA was washed at least two times with 70% ethanol and then desiccated to dryness. The RNA was resuspended in sterile distilled H_2O at 0.5–5.0 μg/μL as described in the text and was used immediately for translational studies or stored frozen at −60 °C. Polysomes were resuspended in nuclease-treated reticulocyte lysate just prior to translational assay.

Reticulocyte lysates were thawed and made exogenous mRNA dependent with micrococcal nuclease (Boehringer Mannheim) as described by Pelham & Jackson (1976) just prior to use with the following modification: nuclease digestion time was increased from 13 to 15 min, which reduced the assay background by 30% without affecting translation of exogenous mRNAs.

The translational system of Strohman et al. (1977) (system I) contained in a final volume of 50 μL: 40% (v/v) nuclease-treated reticulocyte lysate, 80 mM KCl, 2 mM $Mg(C_2H_3O_2)_2$, 1 mM ATP, 0.2 mM GTP, 20 μM 19 amino acids minus methionine, 16 mM creatine phosphate, 10 μg/mL of creatine phosphokinase, 0.67 mM $CaCl_2$, 20 mM Hepes, 6 μCi of [^{35}S]methionine (1000–1200 Ci/mmol; Amersham-Searle), RNA, and any other additions as described in the text.

The translational system as described by Pelham & Jackson (1976) (system II) contained in a 50-μL final volume assay: 72.6% (v/v) nuclease-treated reticulocyte lysate, 0.67 mM $CaCl_2$, 16.8 μM hemin (crystalline equine type III; Sigma), 33.6 μg/mL of creatine phosphokinase, 84 mM KCl, 0.42 mM $Mg(C_2H_3O_2)_2$, 32.6 μM 19 amino acids minus methionine, 8.4 mM creatine phosphate, 6–12 μCi of [^{35}S]methionine, RNA, and any other additions as described in the text.

All reactions were incubated at 30 °C for 1 h and terminated by freezing. To determine total incorporation of labeled amino acid into protein, 5-μL aliquots were removed and placed in 1.0 mL of distilled H_2O to which 0.5 mL of digestion mix (1 M NaOH, 0.5 M H_2O_2, and 1 mg/mL of methionine) was added. The reaction was incubated at 37 °C for 15 min and terminated by chilling. Protein was precipitated by addition of 1 mL of 25% trichloroacetic acid (Cl_3AcOH). The precipitate was collected by filtration on glass fiber disks (Enzo Biochemicals, Inc.), washed with 5% Cl_3AcOH, dried, and counted in 10 mL of OCS scintillation cocktail (Amersham/Searle) in a Beckman LS-200B liquid scintillation counter with an efficiency of 74.6%.

Low-Salt Precipitation of Myofibrillar Proteins. To determine the percentage of total in vitro protein synthesis which represented myofibrillar proteins, an aliquot of the terminated translation reaction was precipitated at low-salt concentrations (Wikman-Coffelt et al., 1973). The precipitated material was resuspended in electrophoresis sample buffer, and an aliquot was removed and precipitated in 10% Cl_3AcOH in the presence of 50 μg of cold carrier rat skeletal muscle MHC. The Cl_3AcOH precipitated material was collected by filtration on glass fiber disks and processed as described above.

Quantitation of the percentage of total in vitro protein synthesis which represented MHC was performed as described for total myofibrillar proteins with the following modification:

an aliquot of the terminated translational reaction was incubated for 1 h at 4 °C in the presence of 6 mM ATP to dissociate the actomyosin complex prior to low-salt precipitation.

$NaDodSO_4$-Polyacrylamide Gels. Electrophoresis in $NaDodSO_4$-polyacrylamide slab gels was performed as described by Laemmli (1970). This is a discontinuous gel system utilizing a 3% stacking gel, a 7% or 10% running gel, and a pH 8.3 electrode buffer. Equal volumes of sample and sample buffer (20% glycerol, 0.125 M Tris-HCl, pH 6.8, 2% $NaDodSO_4$, 5% β-mercaptoethanol, 7 M urea, and 10 mM L-methionine) were mixed and then heated in a boiling H_2O bath for 1 min before applying to the stacking gel. The samples were run into the stacking gel at 30 mA for 30 min, and the gels were run for 9 h at 15 mA.

Fluorography. Gels were impregnated with PPO following the procedure of Bonner & Laskey (1974). The gels were dried overnight with vacuum and heat. Kodak SB-5 X-ray film was presensitized according to the method of Laskey & Mills (1975). Development was for 5 min in Kodak X-ray film developer at 25 °C.

Peptide Analysis. Peptide mapping by limited proteolysis and electrophoresis was carried out by the procedure described by Cleveland et al. (1977) with some modifications.

[^{35}S]Methionine-labeled MHC was prepared from in vitro translational assays by low-salt precipitation in the presence of ATP as described above. [^{3}H]Methylmethionine-labeled MHC was prepared from well-differentiated myotube cultures labeled in vivo with 50 μCi/mL of [^{3}H]methylmethionine (7-10 Ci/mmol; Schwarz/Mann) for 24 h, followed by low-salt precipitation in the presence of ATP as described above. Resuspended [^{3}H]MHC and [^{35}S]MHC were mixed and subjected to electrophoresis on $NaDodSO_4$-polyacrylamide slab gels as described above. The gels were stained with 0.2% Coomassie blue in methanol-acetic acid-H_2O (5:1:5) for 15 min at room temperature. The gels were destained in 10% acetic acid for 30 min at room temperature with gentle agitation. The MHC band was excised, equilibrated with 10 mL of 0.125 M Tris, pH 7.4, 0.1% $NaDodSO_4$, and 1 mM EDTA for 1 h, and stored at −20 °C until used. A slice of the MHC band was placed on top of a $NaDodSO_4$-polyacrylamide disk gel comprised of a 6-cm 10% polyacrylamide resolving gel and a 3.5-cm 3% polyacrylamide stacking gel. The stacking gel contained 1 mM EDTA. The MHC was run 1.75 cm into the stacking gel at 5 mA. Chymotrypsin (0.1-1.0 mg) was applied to the gel. Digestion and subsequent electrophoresis were performed as described by Cleveland et al. (1977). The gels were cut into 1-mm slices using a Mickle gel slicer, depolymerized with 700 μL of 30% H_2O_2 overnight at 37 °C, and counted in 10 mL of Beckman Ready Solv scintillation cocktail in a Beckman LS-200B liquid scintillation counter with a double labeling setting with 5% spillover of ^{35}S counts into the ^{3}H channel and no spillover of ^{3}H counts into the ^{35}S channel.

DEAE-Cellulose Chromatography. MHC was isolated by chromatography over DEAE-cellulose following the procedure of Offer et al. (1973).

Results

A. The Reticulocyte Lysate. A standard rabbit reticulocyte cell-free extract (lysate) (Schimke et al., 1974) was converted into an mRNA-dependent protein synthesizing system by removal of endogenous translatable mRNA by preincubation of the lysate with micrococcal nuclease (Pelham & Jackson, 1976). Lysates treated in such a manner have negligible endogeneous protein synthetic activity yet translate exogenous mRNA with high efficiency.

Table I: Effect of Hemin, ATP, GTP, K^+, Cl^-, Mg^{2+}, and Ribosomal Wash on Total and Myofibrillar Protein Synthesis

additions to the reaction[a]	cpm/5 μL of aliquot: blank	2 μg of poly(A)$^+$ RNA	10 μg of poly(A)$^-$ RNA[b]	5 μg of total RNA	myofibrillar proteins, % of total protein synthesis[c] (%)
system I (original)	1 400	1 200	1 250	1 800	<1
system I + 16.8 μM hemin	1 500	13 300	5 100	6 200	10.1
system II (original)	1 000	11 300	4 600	5 500	13.9
168 mM K^+, 168mM Cl^-	1 300	1 950	1 980	2 800	5.3
168 mM K^+, 84 mM Cl^-	900	45 900	12 400	19 200	23.8
1.0 mM Mg^{2+}	900	32 600	9 400	15 700	18.7
0.2 mM GTP, 0.2 mM ATP	1 400	5 100	2 500	2 900	11.7
0.05 mM GTP, 1 mM ATP	1 250	22 500	10 300	15 500	15.2
system III (168 mM K^+, 84 mM Cl^-, 0.05 mM GTP, 1 mM ATP, 1.0 mM Mg^{2+})	950	55 400	25 850	36 300	38.8
system III + ribosomal wash	1 500	43 300	21 650	26 200	33.1

[a] All additions, except where indicated, were made to 50-μL translational reactions using the reaction conditions as described for system II as the reaction base. [b] Poly(A)$^-$ RNA is defined here as RNA which fails to bind to an oligo(dT)-cellulose column. [c] The values presented represent total myofibrillar protein synthesis directed by total cytoplasmic RNA. The percentage of total protein synthesis which represents myofibrillar proteins was determined by low-salt precipitation as described under Materials and Methods.

Using the nuclease-treated reticulocyte lysate, we compared the complete in vitro protein synthesizing systems of Strohman et al. (1977) (system I) and Pelham & Jackson (1976) (system II) in order to determine the optimum conditions for the synthesis of high molecular weight muscle-specific proteins such as MHC. In contrast to the results reported by Strohman et al. (1977) for system I, little or no protein synthesis was observed in the absence of hemin (Table I). In the presence of hemin, system I was approximately 10-15% more active in the incorporation of labeled amino acids into proteins than was system II. However, the latter system quantitatively favored the synthesis of the higher molecular weight muscle-specific proteins such as MHC (Table I) and was therefore used as the basis for all further studies.

B. Determination of Mg^{2+}, Cl^-, K^+, GTP, and ATP Optima. The optimum conditions for total protein and for myofibrillar protein synthesis were determined (Table I). Addition of 1 mM ATP and 0.05 mM GTP to the reaction increased the total incorporation by twofold for poly(A)$^+$ RNA and by eightfold for total RNA. A sharp Mg^{2+} optimum was observed at 1.0 mM. Total incorporation was increased 2.7-fold. None of these additions, however, significantly enhanced the synthesis of myofibrillar proteins. Two K^+ optima were observed, one at 84 mM K^+ and the other at 168 mM K^+. At the second optimum, 168 mM, total incorporation was increased three- to fourfold over that observed at 84 mM K^+. In addition there was a significant increase in the pro-

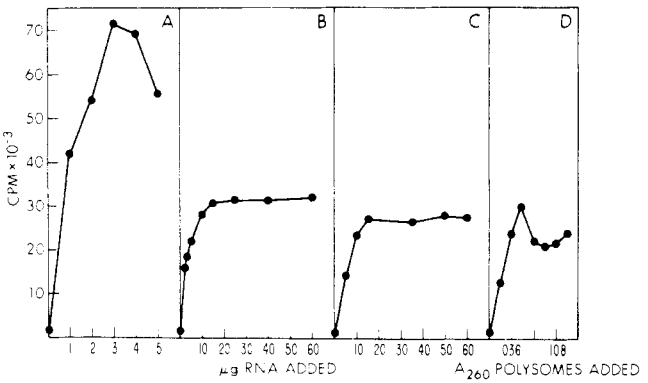

FIGURE 1: Effect of different RNA additions on total protein synthesis in system III. System III was developed as described in the text. Incubations were for 60 min at 30 °C. The ordinate represents the number of counts incorporated in a 5-μL aliquot of a 50-μL translational reaction. (A) Poly(A)$^+$ RNA isolated by oligo-(dT)-cellulose chromatography; (B) total cytoplasmic RNA; (C) total cytoplasmic RNA heated at 65 °C for 5 min and quick cooled just prior to addition to the translational reaction; and (D) total polysomes prepared as described under Materials and Methods and resuspended in nuclease-treated reticulocyte lysate just prior to addition to the translational reaction.

duction of myofibrillar proteins. However Cl^- concentrations greater than 84 mM strongly inhibited protein synthesis and decreased the relative proportions of myofibrillar proteins synthesized. In order to prevent the addition of excess Cl^-, K^+ in excess of 84 mM was added as the acetate salt.

The conditions that individually increased total incorporation were combined (system III) (Table I). Exogenous RNA-directed incorporation was increased five- to nine-fold over that of the original reaction conditions. More importantly, the synthesis of high molecular weight muscle-specific proteins, i.e., MHC and associated polypeptides, was greatly enhanced (Table I). Finally, addition of varying amounts of a ribosomal wash which is reported to stimulate translation (for a review, see Anderson et al., 1977) neither increased total incorporation nor enhanced MHC synthesis (Table I).

C. Determination of Optimum Template Concentration. Optimum exogenous RNA concentrations for maximal protein synthesis were determined (Figure 1). Maximum incorporation is observed with 3 μg of poly(A)$^+$ RNA from any source; addition of more poly(A)$^+$ RNA is inhibitory (Figure 1A). Inhibition of total incorporation in the reticulocyte system by high concentrations of exogenous RNA has been reported previously (Schimke et al., 1974). In contrast, addition of 10 μg of total cytoplasmic RNA gives maximum incorporation of label, but the addition of up to 60 μg of total RNA is not inhibitory to this system (Figure 1B). Heating and quick cooling of the RNA to eliminate secondary structure do not increase its ability to direct the incorporation of labeled amino acids into protein; the level of incorporation directed by treated RNA was similar to that with RNA that was not heated (Figure 1C). In addition, this system has the capability of translating total polysomes obtained by centrifugation through a 1 M sucrose pad without any further purification of the RNA contained therein (Figure 1D). Finally, RNA that fails to bind to an oligo(dT)-cellulose column is also capable of directing protein synthesis in this system (Table I). It is interesting to note that total RNA and RNA that fails to bind to an oligo(dT)-cellulose column are more efficient in translation than poly(A)$^+$ RNA when corrected for probable mRNA content. The reason for these differences is not clear at this time, but it is possible that differentiated L_6E_9 cells contain a larger amount of mRNA which fails to bind to

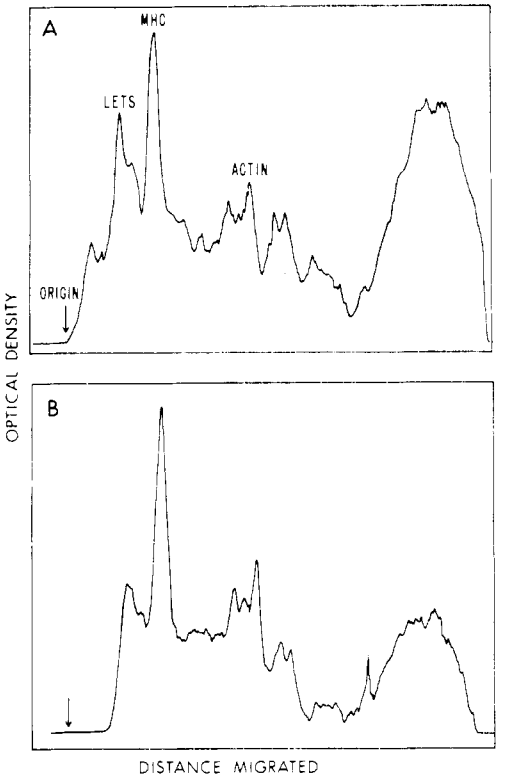

FIGURE 2: Comparison of the profiles of total proteins synthesized in vivo and in vitro. Densitometer scan of a fluorograph of a 10% polyacrylamide gel prepared as described under Materials and Methods. (A) Total proteins from well-fused L_6E_9 cells labeled for 3 h in vivo with [^{35}S]methionine; (B) total protein, labeled with [^{35}S]methionine, synthesized in vitro in system III by 5 μg of total cytoplasmic RNA extracted from well-fused L_6E_9 cells. System III displays a high degree of fidelity with regard to the range and relative proportions of proteins synthesized as compared to the proteins synthesized in vivo by the cells from which the RNA was obtained. The relative positions of LETS (M_r ~250 000), MHC (M_r ~200 000), and actin (M_r ~41 000) synthesized in vivo and in vitro are shown.

oligo(dT)-cellulose than has been reported for other cells.

Under conditions of nonsaturating RNA concentration, protein synthesis proceeds in a linear fashion for at least 2 h (data not shown). More importantly, using unfractionated RNA preparations this system exhibits a striking degree of fidelity with regard to the types and relative proportions of proteins synthesized as compared to the pattern of proteins synthesized by the intact cell from which the RNA was obtained (Figure 2). Under the final assay conditions, the relative proportion of myofibrillar proteins synthesized as a percentage of total protein synthesis approaches 40% (Table I), which is similar to the in vivo myofibrillar synthesis of the fully differentiated myotube.

D. Identification of the in Vitro Synthesized 200 000-Dalton Protein as True MHC. Figure 2 demonstrates that a protein with the mobility of MHC is one of the major proteins coded by total RNA from fused myotubes. This protein can be specifically precipitated by lowering the salt concentration in the presence of ATP (see Materials and Methods). Under these conditions, only MHC is precipitated (data not shown). After precipitation it comigrates with marker MHC (prepared by either ionic precipitation or by DEAE-cellulose chromatography) on different percentage polyacrylamide gels (Figure 3). Moreover, a limited peptide analysis by the procedure of Cleveland et al. (1977) shows complete identity with in vivo labeled MHC (Figure 4). Thus, even though the existence of some heterogeneity in the molecule or small differences between the in vivo and in vitro synthesized MHC cannot be

Table II: Comparative Translation of Muscle Cell RNA[a]

cell type	system III					system I				
	total incorporation	cpm in myofibrillar proteins[b]	% myofibrillar proteins	cpm in MHC[c]	% MHC	total incorporation	cpm in myofibrillar proteins[b]	% myofibrillar proteins	cpm in MHC[c]	% MHC
growing myoblast	332 790	118 470	35.6	54 910	16.5	259 800	9 871	3.8	1 039	0.4
differentiated myotube	275 700	107 247	38.9	51 010	18.5	152 700	47 640	31.2	25 655	16.8

[a] Each 50 μL translation reaction contained 5 μg of total cytoplasmic RNA from the same RNA preparation, and the reactions were performed on the same day with the same batch of reticulocyte lysate. [b] Myofibrillar protein synthesis was quantitated by low-salt precipitation as described under Materials and Methods. [c] MHC synthesis was quantitated by low-salt precipitation following dissociation by ATP as described under Materials and Methods.

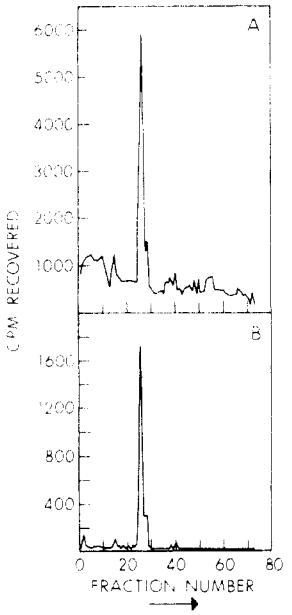

FIGURE 3: Polyacrylamide gel electrophoresis of MHC purified by low-salt precipitation. MHC was purified by low-salt precipitation following ATP dissociation of the actomyosin complex as described under Materials and Methods. Low-salt-precipitated MHC was then run on an 8-cm 7% polyacrylamide gel, and the gel was processed as described under Materials and Methods. (A) In vivo [^{3}H]-methylmethionine-labeled MHC isolated from well-fused L_6E_9 cells; (B) in vitro [^{35}S]methionine-labeled MHC isolated from total proteins synthesized in system III using total cytoplasmic RNA extracted from well-fused L_6E_9 cells.

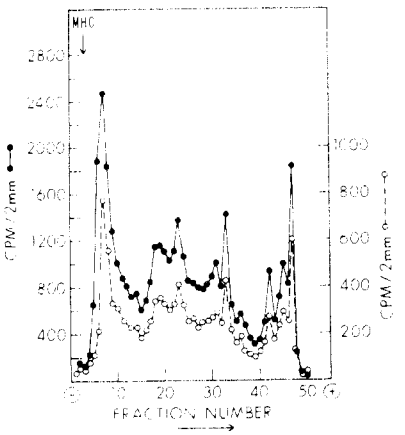

FIGURE 4: Peptide mapping of in vivo and in vitro synthesized MHC by limited proteolysis. Peptide mapping by limited chrymotropic digestion was performed by a modification of the procedure of Cleveland et al. (1977). MHC was purified by low-salt precipitation in the presence of ATP, followed by polyacrylamide gel electrophoresis as described under Materials and Methods. In order to obtain significant proteolysis of MHC it was necessary to run the MHC out of the same gel and halfway into the stacking gel before adding the proteolytic enzyme. If the MHC and the enzyme were added together, no proteolysis was observed because MHC is a large slow-moving molecule and runs behind the enzyme in the stacking gel. Digestion was for 1 h at room temperature. In the figure shown above, 500 μg of chymotrypsin was used. If the digestion time is kept constant, 0.1–1.0 mg of chymotrypsin can be applied. The same peaks appear at all enzyme concentrations although their relative ratios change. No new peaks appear at higher enzyme concentrations. The pattern of peaks appearing is unique to MHC. Other myofibrillar proteins treated in a similar manner produced different peptide patterns (data not shown). The arrow in the figure represents the position of intact undigested MHC. (●) In vivo [^{3}H]methylmethionine-labeled MHC isolated from well-fused L_6E_9 cells. (○) [^{35}S]methonine-labeled MHC isolated from proteins synthesized in vitro in system III using 10 μg of total cytoplasmic RNA from well-fused L_6E_9 cells.

excluded by the procedures used, all the available evidence supports the conclusion that the in vitro synthesized 200 000-dalton protein is truly MHC.

E. Comparative Translation of MHC mRNA from Different Stages of Muscle Differentiation. Cell-free in vitro translational systems are widely used to determine whether changes in rate of protein synthesis correlate with changes in mRNA levels when a specific cDNA probe to titrate the mRNA is not available. The results shown in Table II suggest that this approach is not always valid. In growing L_6E_9 cells MHC synthesis represents <1% of total protein synthesis but represents as much as 20% 2 days after fusion, when >90% of nuclei are found in myotubes. We were interested in determining whether this increase in MHC synthesis was correlated with a similar increase in MHC mRNA levels during differentiation. Table II shows that, when different translational systems are used, opposite results can be obtained using the same RNA preparations. When RNA from growing and fused cells is translated in the system described here (system III), both stimulate the synthesis of MHC to very similar levels, implying that MHC mRNA is present in growing myoblast. When the same RNAs are translated under the conditions described by Strohman et al. (1977) (system I), the RNA from growing myoblasts does not induce MHC synthesis but the RNA from fused myotubes is highly efficient in MHC synthesis. Thus, the failure of the RNA from growing cells to induce MHC synthesis in system I cannot be due to a general inability of the system to translate MHC mRNA. In system I, it seems that MHC induction during differentiation correlates with MHC mRNA accumulation, while with our conditions it would seem that MHC mRNA levels do not correlate with in vivo MHC synthesis. These same results have been obtained with five different RNA preparations translated in the two systems. The MHC coded for by RNA from growing cells in our system is indistinguishable from true MHC by size, precipitation in low salt, and peptide analysis. However, myoblast mRNA must be qualitatively different from myotube mRNA because of its different behavior in the different translational systems. Whether the difference is in the MHC mRNA or in some

Table III: Translation of RNA from NonMuscle Sources

source of RNA[a]	system III total incorporation	system III % myosin-like peptides	system I total incorporation	system I % myosin-like peptides
Friend erythroleukemia clone 745	21 130	<1.0	15 830	<1.0
rat liver	45 000	1.3	27 750	1.1
hepatoma HTC	274 450	<1.0	86 070	1.9
MuLV	81 110	<1.0	18 860	<1.0

[a] Total cytoplasmic RNA prepared as described under Materials and Methods.

other RNAs is not known at this time. Nonetheless it is clear that different translational systems can give qualitatively different results even when the same RNA preparations are used.

F. RNAs from Nonmuscle Sources Do Not Synthesize Detectable Amounts of MHC. To determine whether the synthesis of MHC in our system by mRNA from growing myoblasts was a muscle-specific property and was not due to some poorly understood property of the translational system, cytoplasmic RNA from different nonmuscle sources was tested under the same conditions used to translate RNA from L_6E_9 cells. As shown in Table III, nonmuscle RNA tested induced very low levels of myosin-like molecules when translated either in system III or I.

Discussion

We have developed a translational system which we have used to determine the relative levels of translatable MHC mRNA in dividing myoblasts which do not accumulate MHC and in fused myotubes where MHC may represent as much as 20% of the total proteins synthesized. In our system MHC mRNA is observed to be present in RNA preparations from growing cells that in other translational assays did not direct MHC synthesis. By our assay, dividing myoblasts appear to contain the same relative amount of translatable MHC mRNA as do fused myotubes. However, the MHC mRNA present in dividing myoblasts must, in some manner, be qualitatively different from myotube MHC mRNA. The discrepancy in the results reported on MHC regulation during muscle differentiation (John et al., 1977; Patterson & Bishop, 1977; Strohman et al., 1977; Bag & Sarkar, 1976; Robbins & Heywood, 1978) could be due to the different assays used to detect MHC mRNA. The reasons for this discrepancy are not clear at this point but our preliminary results using purified MHC cDNA (Benoff & Nadal-Ginard, unpublished results) suggest that differences in poly(A) content in MHC mRNA during myoblast differentiation are at least partially responsible for differences in translational efficiency. Furthermore, Heywood & Kennedy (1976) have presented data suggesting that myoblast MHC mRNA is sequestered in a manner that inhibits its translation. We also have data which suggest that the failure of dividing myoblasts to accumulate MHC is due, in part, to the presence of a translational inhibitor (Benoff & Nadal-Ginard, unpublished results). These results emphasize that translational assays, at least for very large molecules, give only qualitative results. Moreover, the results are meaningful only when positive data are obtained. Negative results, that is, failure to translate a given molecule, should be interpreted with caution since, as proven here, they do not always imply that this mRNA is not present.

The in vitro protein synthesizing system described here is especially useful for the translation of mammalian mRNAs that specify proteins of molecular weight greater than 100000. Mammalian proteins as large as LETS (M_r ~240 000) are synthesized in this system. This is the first translational assay in which large mammalian proteins are synthesized in relative proportions similar to those observed in the intact cell. The main feature of our system is the use of K^+ and Cl^- concentrations similar to those of the reticulocyte cytoplasm (Weber et al., 1977a). Our system employs a very high K^+ concentration (168 mM) relative to other published systems. High K^+ levels favor protein-chain elongation (for a review, see Anderson et al., 1977). High K^+ concentrations also favor translation of capped over uncapped mRNAs (Weber et al., 1977b). Therefore, it appears that the synthesis of complete polypeptide chains as well as the preferential translation of capped mRNAs is responsible for the high degree of fidelity of protein synthesized in vitro in our system as compared to in vivo protein synthesis. Since the ability to translate MHC mRNA greatly increases as the K^+ concentration of the translation reaction is raised, our results tentatively suggest that the mRNA for MHC is capped.

An important feature of our translational system is the ease with which the MHC synthesized in vitro can be detected and quantitated. In this system the exogenous mRNA-directed in vitro synthesis of MHC is detected directly by gel electrophoresis of the translation products. Detection of MHC by gel electrophoresis in all previous studies required specific immunoprecipitation of the MHC to separate it from reticulocyte myosin-like polypeptides. Preincubation of the lysate with nuclease prevents the synthesis of most reticulocyte proteins, including those polypeptides with molecular weights similar to that of MHC. We have identified the in vitro translation product as being identical with authentic MHC by comigration with MHC synthesized in vivo on polyacrylamide gels and by low-salt precipitation. MHC precipitates in low salt because the rod portion of the molecule is highly hydrophobic. ATP induces the dissociation of MHC from the other myofibrillar proteins. In the presence of ATP, only MHC precipitates at low-salt concentrations. Other myofibrillar proteins, including actin and myosin light chains will coprecipitate with MHC in low salt only when they are assembled into complete actomyosin molecules. Actin and myosin light chains are found in undissociated low-salt precipitates of the in vitro translation products (data not shown), indicating that the actomyosin complex is assembling in vitro. Furthermore, we have demonstrated the homology of the peptides obtained by limited chymotryptic digestion of the in vitro product with those obtained by digestion of authentic MHC. Previous studies (Sarkar et al., 1973; Morris et al., 1972) have demonstrated the fidelity of MHC synthesized in vitro with authentic MHC. But the major advantage of our assay for the study of MHC synthesis in vitro is that measurable amounts of MHC are synthesized and the relative proportion of MHC synthesis parallels that of the fully differentiated myotube.

Since mRNAs from nonmuscle sources such as mouse myeloma, lymphoma, and Friend and rat hepatoma cells and rat liver, as well as mammalian RNA viruses and their mRNAs, are efficiently and authentically translated in this system (data not shown), the translational assay described here may be useful in the detection of large mRNAs when a chemical titration is not possible or the biological activity of the mRNA has to be tested.

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α- and β-Glycopyranosyl Phosphates and 1,2-Phosphates. Assignments of Conformations in Solution by ^{13}C and ^{1}H NMR†

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ABSTRACT: The ^{1}H and ^{13}C NMR parameters of the anomeric pairs of aldopyranosyl phosphates and their rigid 1,2-phosphate derivatives are reported. The derivatives of D-glucose, D-galactose, and D-mannose exist in the $^{4}C_{1}$ conformation while the L-fuco derivatives are in the $^{1}C_{4}$ conformation. As judged by ^{31}P–^{1}H and ^{31}P–^{13}C coupling constants, all of the α anomers of the aldopyranosyl phosphates have the phosphate moiety predominantly trans to C(2) while in the β anomers other rotamers make significant contributions. This relationship remains the same for the biologically important nucleoside diphosphate sugars (UDPGlc, UDPGal, GDPMan, and GDPFuc). From the pH dependence of ^{13}C chemical shifts, observed in 0.5 M solutions, the pK'_{a2} of the α anomers is 6.1 while the pK'_{a2} of the β anomers is 0.6–0.8 pH unit lower. In the 1,2-phosphates, the chair conformation of the parent aldose is retained while an envelope conformation is formed by the cyclic phosphate. In the α anomers, the plane is formed between C(2), C(1), O(1), and P while O(2) is above the plane. In the β anomers, O(1) is out of the plane formed by the other atoms. The β anomers have phosphorus coupled to C(3) with coupling constants of 10.8–11.7 Hz, approximately 2 Hz greater than the maximum reported for trans coupling (Lapper, R. D., & Smith, I. C. P. (1973) *J. Am. Chem. Soc. 95*, 2880).

Glycosyl phosphates are precursors or components of nucleoside diphosphate sugars, glycoproteins, glycolipids, and complex carbohydrates. Their involvement in biological processes has made the elucidation of their structures, reactivities, and conformations in aqueous solution relevant to studies of structure–function relationships in many biochemical systems.

Few studies of glycosyl phosphate conformations have been reported. Sarma et al. (1973) and Lee & Sarma (1976) have examined the conformation of several glycosyl phosphates and nucleoside diphosphate sugars by ^{1}H NMR and have shown that in the most abundant conformer the phosphate group is positioned trans to C(2) of the pyranosyl ring or bisects the angle between H(1) and C(2). Bundle et al. (1973) examined α- and β-2-acetamido-2-deoxy-D-glycopyranosyl phosphates and polymers derived from them by ^{13}C NMR and concluded that the phosphorus atom was trans to C(2) in all cases. It appears that, in these systems, both homonuclear and heteronuclear vicinal couplings follow a Karplus type of angular dependence, which allows certain conclusions to be drawn with respect to conformation although the exact relationships of coupling to dihedral angle have not been established.

The present study was undertaken to examine the effects of configurational changes, anomeric form, and pH on the NMR parameters of the commonly occurring α-glycosyl phosphates of D-glucose, D-mannose, D-galactose, and L-fucose. In addition, the less common β anomers and the 1,2-phos-

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